ABSTRACT

Polymeric micro-networks architecture (PMNs) based on chitosan and its graft copolymer with acrylamidoglycolic acid (AGA) by precipitation and cross-linking methods for the controlled release of 5-fluorouracil (5-FU) is described. The chitosan and its graft copolymers are cross-linked with different ratios of urea-formaldehyde (UF) in the presence of acidic alcohol media at room temperature. The networks are characterized by Fourier transform infrared spectroscopy, particle size analysis, scanning electron microscopy, differential scanning calorimetry, and X-ray diffraction studies. The physicochemical characteristics (i.e., encapsulation efficiency, density, particle size distribution, in vitro release) of the PMNs are reported. The extent of cross-linking is studied in terms of size of PMNs as well as their release characteristics. Extent of drug loading on the encapsulation efficiency of the microparticles is investigated to prove their linear relationship. The in vitro release performed in 1.2 pH solution followed by 7.4 pH buffer media, has been analyzed with an empirical equation to understand the diffusion nature of drug solution through the PMNs. The study of 5-FU loaded PMNs shows that the extended release rates are noticed from the conventional dosage release duration beyond 18 h.

INTRODUCTION

The main advantage of natural polymers lies in their biocompatibility and biodegradability performance, without producing systemic toxicity upon drug administration [1,2]. Modification of polymers has received great practical and academic interest for the controlled release (CR) of drugs and proteins [3-7], since they provide a convenient route for the modification of properties to meet the specific needs. In recent years considerable interest has been given to the development of polymeric networks (PN) [8-12] owing to their biomedical applications due to their wide range of mechanical properties and transformation potentials. These superior properties allow a variety of different shapes with different sizes (i.e., from centimeter to nanoscales) of PNs to be easily obtained at low cost production. The micro/nano particulate drug delivery systems offer numerous advantages over the conventional dosage forms; which include higher efficacy, reduced toxicity.
and improved patient compliance [13-16]. Oral controlled release is capable of multiple unit dosage forms such as beads, pellets and microparticles which are more popular than single unit dosage forms due to several inherent advantages [17-19].

Natural polysaccharides, e.g., chitosan (CS), pectin, guar-gum, alginates, dextran, amylose, xanthan gum and chondroitin sulphate are promising alternative polymers, but suffer from hydrolysis of their glycosidic bonds in colon. The only drawback of these polymers is their high solubility in the gastrointestinal tract fluids that implies the need of cross-linking to assess their integrity until they reach the colonic region [20-22]. pH-Dependent release can be assured by enteric coating and drug release can be delayed further for a predetermined time during transit through the small intestine. Time-controlled release systems may be swellable with soluble coatings, or a matrix type, which can resist the complete drug release from the whole administration intake for additional 3 h (i.e., the usual small intestinal transit time) and deliver the drug specifically to the colon. Conventionally, various polysaccharides/polymers are used in the tablet formulations to slow the drug release. Among these polysaccharides, chitosan and its derivatives have generated significant attraction due to their specific structure and superior physicochemical properties, which lead to excellent biocompatibility, biodegradability, low immunogenicity, and biological activities [23,24].

Further, this polymer can be used as a carrier for colon-selective delivery of drugs. This application is largely based on its specific biodegradability by the enzyme, lysozyme, which is highly concentrated in the mucosa, and by the enzymes secreted by the colonic bacteria [25,26]. In addition, CS can be used as an ingredient in food materials, which suggests its acceptability as a good excipient for oral administration [27,28]. Despite these promising characteristics, a slight drawback of CS is its rapid dissolution in the gastrointestinal tract conditions. Chemical cross-linking with aldehydes and blending with synthetic polymers has been used to overcome this problem [29-31]. Nevertheless, the toxicity of aldehydes enormously limits the exploitation of these cross-linked microcapsules. In general, UF resins are rigid, however, in case of networks containing natural polysaccharides they are flexible and slowly degradable polymers. These amino resins have been used as cross-linkers for membranes, CR of pharmaceutical and nonpharmaceutical agents such as pesticides [32-34]. The advantage of a UF cross-linker is that it can be easily prepared at room temperature. Moreover, the properties of the polymer can be altered during the process of polymerization.

5-Fluorouracil (5-FU) is most commonly used as an anti-cancer drug for the treatment of solid tumors of breast, stomach, colon and pancreas [35-38]. It has been widely used in drug administration in spite of its large number of secondary effects that accompany its conventional administration. However the drug exhibits very high toxicity with many side effects [39]. Stomach- and site-selective drug delivery is a very important strategy for the optimization of chemotherapy in terms of efficacy and safety. The gastric serosal surface application of anticancer drugs holds promise for the site-selective delivery in the stomach. To the best of our knowledge there are no reports on 5-FU loaded chitosan PMNs.

This work reports the development of a novel 5-FU loaded chitosan based particulate PMNs cross-linked with urea-formaldehyde obtained by a simple one step method. These matrices are intended to enhance the mechanical properties and the degradation kinetics of the polymer micro-networks in the phosphate buffered solution. The networks formed have been characterized by Fourier transform infrared spectroscopy, particle size analysis, scanning electron microscopy, differential scanning calorimetry, and X-ray diffractometry. The in vitro release studies have been performed in 1.2 pH solution for 1 h, followed by 7.4 pH buffer solution at 37°C.

In this study, the novelty of our consideration is mainly based on the UF cross-linked chitosan and its copolymers with PAGA to develop potentials PMNs. The developed PMNs were used for controlled release of 5-FU for 18 h administration doses. Moreover, to the best of our knowledge, studies on UF and chitosan PMNs encapsulated with 5-FU have not been reported yet.
EXPERIMENTAL

Materials
5-Fluorouracil (5-FU), acrylamidoglycolic acid (AGA), and chitosan (CS, low molecular weight with degree of deacetylation of 84%) were purchased from Aldrich, Milwaukee, USA. Urea, formaldehyde, sulphuric acid, glacial acetic acid and ethanol were purchased from Alfa Aesar, Tokyo, Japan and were used without further purification.

Preparation of Drug Loaded PMNs
To a 1% solution of chitosan in 2% aqueous acetic acid prepared by gentle heating and stirred with a stirrer on a hot plate, a required amount of drug was added until a complete dispersion of the drug in the polymer solution was obtained. A portion of 5 mL of the drug loaded polymer solution was added dropwise in a short time of 50 to 75 s into the ethanol solution containing the required amount of cross-linking agent (UF), followed by the addition of 1.12 M H₂SO₄ into the mixture. PMNs formed were removed from ethanol at a particular time and were repeatedly washed with distilled water to remove any unreacted material. Totally, eight formulations were prepared by varying the amount of drug, and UF ratios with chitosan matrices. The grafting of AGA onto the chitosan backbone (Scheme I) was carried out by a persulphate-induced free-radical reaction [40]. Proposed structure of UF cross-linked CS is shown in Scheme II. The copolymeric microparticles were prepared by the same conditions mentioned above.

Density Measurement of PMNs
The product, PMNs, was taken in specific gravity bottles in order to determine its density. The amount of water displaced from the specific gravity bottle was considered to be the volume of the water minus the filled capsules. The density of the capsules was then calculated as the ratio of the weight of the capsules to its total volume.

Entrapment Efficiency and Dissolution Tests
Entrapment efficiency and dissolution tests were carried out as reported procedure [41].
Fourier transform infrared spectroscopy (Nicolet, Impact 410, USA) analysis was performed to identify the chemical structure of the PMNs. Furthermore, to investigate the drug nature in the PMNs, differential scanning calorimetry (DSC) (Model-DSC SP, UK) analysis was performed for 5-FU, 5-FU loaded hydrogels, and pristine hydrogel with a scanning rate of 10°C/min and nitrogen gas flow rate of 50 mL/min. To support these DSCs results, X-ray diffraction analysis was performed with general area detector diffraction system (Philips, X'pert-PW3040, Netherlands) using a CuKα radiation. Morphology of the PMNs was examined by scanning electron microscopy (SEM) (JSM- 5610, JEOL, Japan). Particle size of the PMNs was measured by using a particle size analyzer (Mastersizer 2000, Malvern Instruments, UK).
RESULTS AND DISCUSSION

FTIR Studies
Graft copolymer based on chitosan has been synthesized by grafting AGA onto polysaccharide in aqueous medium using potassium persulphate as an initiator. The grafting was confirmed by comparing the FTIR spectra of chitosan (Figure 1 spectrum a) with that of the grafted product (Figure 1 spectrum b) and CS. The FTIR spectrum of the CS has a strong peak around 3400 cm\(^{-1}\) due to the stretching vibration of O-H, the extension vibration of N-H, and intermolecularly hydrogen bonds of the polysaccharide. Given the fact that the stretching vibration of amino groups causes a band at 3475 cm\(^{-1}\) and the O-H stretching vibration of the graft copolymer (Figure 1 spectrum b) shift to a lower wavenumbers compared to those of the CS (Figure 1 spectrum a) (due to overlapping of O-H stretching of chitosan and N-H stretching of amide groups at PAGA grafts). Reduced intensity of this peak with respect to that of chitosan shows that appreciable amount of O-H and N-H groups on chitosan chains have been successfully grafted with poly(acrylamidoglycolic acid) chains. The very intense characteristic band at 1560 cm\(^{-1}\) is due to the C-O asymmetric stretching vibration of the carboxyl anion from PAGA grafted on CS. This is reconfirmed by the other sharp peak at 1400 cm\(^{-1}\), which is related to the symmetric stretching vibration mode of carboxyl anion [42]. Amide-I and amide-II bands are observed at 1671 and 1629 cm\(^{-1}\), however, these peaks are seen masked with sharp peak present in the chitosan spectrum around 1578 cm\(^{-1}\) which in graft copolymer, it is seen at 1583 cm\(^{-1}\) (Figure 1 spectrum b). Peak around 1432 cm\(^{-1}\) due to C-N stretching vibration in graft copolymer further supports the grafting.

UF was found to be responsible for cross-linking the -NH\(_2\) groups of chitosan in the presence of sulphuric acid. Figure 2 displays the FTIR spectrum of cross-linked PMNs of CS. The increase in intensity and the shift of bands to a higher wavenumber in the region of 1200-1500 cm\(^{-1}\) are indicative of the increase in the number of -CH\(_2\) groups [43] in the cross-linked PMNs. The large increase in this band results from the large increase in the number of C-O bonds, as well as the cross-linking of the PMNs. A strong peak around 1120-1130 cm\(^{-1}\) is assigned to the formation of ionic bond between two chitosan chain molecules. The band appeared at 702 cm\(^{-1}\) is due to C-O-S bond of the cross-linked chains, indicating the reaction between HOSO\(_3^-\) and methylolurea. The FTIR spectra confirmed the successful cross-linking of PMNs with UF in the presence of sulphuric acid.

Morphology and Particle Size
As shown in Scheme II, CS-PMNs were prepared by the ionic interactions between positively charged amino groups of chitosan and negatively charged counterions of urea-formaldehyde-sulphuric acid. Moreover, chitosan is a weak polybase, and as pH of the solution decreased, the ionization of the amine groups of chitosan increased. As shown in Figures 3a and 3b, the surface morphologies of CS-PMNs are...
spherical in shape, and possesses a rough surface [44,45]. These results show that the formation of CS-PMNs matrix is dependent on pH values of the UFS solution, and also it may be due to the molecular weight of CS. Rough and folded surfaces of the PMNs may be due to the lower pH, as the chains come closer to each other and give a regular fibrous structure. Results of the mean particle size with standard errors are presented in Table 1, while the size distribution curve for a typical formulation containing 0.5 UF ratio, 15% UF (MS-2 sample) is presented in Figure 4. The size distribution is bell-shaped (normal distribution) showing 0.91 μm as the mean particle diameter.

Table 1. Results of encapsulation efficiency, mean size and density of CS-UF MSPs loaded with 5-fluorouracil.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>CS (g)</th>
<th>Amount of AGA (wt%)</th>
<th>UF ratio</th>
<th>Drug loading (wt%)</th>
<th>Mean particle size (μ)</th>
<th>Encapsulation efficiency (%)</th>
<th>Density of MPs (g/cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS-1</td>
<td>0.50</td>
<td>-</td>
<td>0.25</td>
<td>15</td>
<td>0.57±0.6</td>
<td>52±0.7</td>
<td>1.479</td>
</tr>
<tr>
<td>MS-2</td>
<td>0.50</td>
<td>-</td>
<td>0.50</td>
<td>15</td>
<td>0.91±1.1</td>
<td>58±0.5</td>
<td>1.397</td>
</tr>
<tr>
<td>MS-3</td>
<td>0.50</td>
<td>-</td>
<td>1.00</td>
<td>15</td>
<td>1.63±1.4</td>
<td>67±1.2</td>
<td>1.286</td>
</tr>
<tr>
<td>MS-4</td>
<td>0.50</td>
<td>-</td>
<td>0.50</td>
<td>05</td>
<td>0.86±0.5</td>
<td>63±2.3</td>
<td>1.481</td>
</tr>
<tr>
<td>MS-5</td>
<td>0.50</td>
<td>-</td>
<td>1.00</td>
<td>25</td>
<td>2.61±1.2</td>
<td>70±1.1</td>
<td>1.361</td>
</tr>
<tr>
<td>MS-6</td>
<td>0.50</td>
<td>10</td>
<td>0.50</td>
<td>15</td>
<td>3.71±0.9</td>
<td>61±1.3</td>
<td>1.465</td>
</tr>
<tr>
<td>MS-7</td>
<td>0.50</td>
<td>20</td>
<td>0.50</td>
<td>15</td>
<td>5.13±0.5</td>
<td>68±1.7</td>
<td>1.411</td>
</tr>
<tr>
<td>MS-8</td>
<td>0.50</td>
<td>30</td>
<td>0.50</td>
<td>15</td>
<td>7.82±2.1</td>
<td>76±0.6</td>
<td>1.388</td>
</tr>
</tbody>
</table>
**Figure 5.** DSC curves of: (a) plain CS-UF PMNs, (b) 5-FU loaded CS-UF PMNs, and (c) pure 5-FU.

**Differential Scanning Calorimetry Analysis**

DSC thermograms of plain CS microparticles, 5-FU loaded CSUF microparticles, and pure 5-FU are displayed in Figure 5. The DSC curves of pure 5-FU show a sharp peak at 285.16°C due to polymorphism and melting, but in case of 5-FU-loaded microparticles, no characteristic peak was observed at 285.16°C, suggesting that 5-FU is molecularly dispersed in the CSUF matrix [8-11].

**X-ray Diffraction Studies**

XRD studies help to find the crystallinity of drug in the cross-linked network PMNs. The XRD patterns of plain CS-PMNs, 5-FU loaded CSUF-PMNs, and pure 5-FU are compared in Figure 6. The XRD of the placebo UFS cross-linked CS-PMNs exhibit the peaks at 2θ of 23°, 38°, and 45°. These peaks are similar to the drug loaded CS-PMNs. The most intensive peaks of 5-FU are observed at 2θ of 29° and 32°, suggesting its crystalline nature. But, these peaks are not found in 5-FU loaded CS-PMNs, indicating that drug is being dispersed at the molecular level in the polymer matrix [8-11].

**Density of the UF Matrix**

The densities of the CS matrix (Table 1) are found to vary between 1.2857-1.4787 g/cm³. These values depend on the ratio of UF, the amount of drug encapsulated and the copolymer content. At the UF ratio of 0.25, the matrix was found to be more rigid and highly dense compared to the UF ratio of 1.0. The density decreased with an increase in the amount of the AGA. This might be due to an increase in volume by increasing the loading, as the polymer becomes less rigid at higher levels of encapsulation.

**In Vitro Drug Release Kinetics**

Numerous mathematical models describing drug release from HPMC-based controlled release formulation have been developed [46]. The most important aspect in developing new pharmaceutical products or evaluating drug release mechanisms gives suitable predictability and accuracy of the model. In many cases, the use of simple empirical or semi-empirical models such as classical Higuchi equation and the so-called power law is fully sufficient. Drug release kinetics were analyzed by plotting the cumulative release data, \( \frac{M_t}{M_\infty} \) versus time by fitting the data to a simple exponential equation [46]:

\[
\left( \frac{M_t}{M_\infty} \right) = kt^n
\]

where \( M_t \) corresponds to the amount of drug released in time \( t \), \( M_\infty \) is the total amount of drug that must be released at infinite time, \( k \) is a constant and \( n \) is the release exponent indicating the type of drug release mechanism. For example, \( n = 0.49 \) for case I or Fickian diffusion, which is characterized by a dependence on the square root of time in both the amount...
diffused and the penetrating diffusion front position; 
$n = 0.89$ for case II transport, which is completely governed by the rate of polymer relaxation and exhibits a linear time dependence in both the amount diffused and penetrating swelling front position; $0.49 \leq n < 0.89$ for anomalous behaviour or non-Fickian transport, which is exhibited whenever the rates of Fickian diffusion and polymer relaxation are comparable [47]. These results along with correlation coefficients, $r$, are presented in Table 2.

Values of $k$ and $n$ have shown system’s dependency on the extent of cross-linking and percentage of drug loading in the CS matrix. The values of $n$ for PMNs prepared by varying the UF ratios, with constant percentages of drug which range from 0.4346 to 0.5282 (Table 2) show the quasi-Fickian type transport mechanism. This indicates that the interactions between the PMNs and dissolution medium increase with the addition of the amount of UF ratio, drug and copolymer contents in the PMNs. And also, it may be due to the swelling capacity of the PMNs which increases with elevation of hydrophilic copolymer content in a counter ion medium with higher pH values.

5-FU is one of the most common antineoplastic drugs used for the treatment of several malignancies. Owing to its high toxicity, it is a good candidate for controlled release technology in order to obtain a therapeutic effect in situ and minimize collateral effects of the drug. The in vitro release profiles of 5-FU from PMNs containing 1 (w/v%) CS, 5, 15 or 25 (w/w%) 5-FU and 0.25, 0.50 or 1.00 UF ratios, respectively in constant amount of aqueous sulphuric acid media were investigated in phosphate buffer pH 7.4.

**UF Effect**
The presence of urea formaldehyde (UF) is an important factor which affects the drug release from the formulations, as the rigidity of the CS matrix is controlled by UF. The higher release rates were observed for 5-FU from the particles produced at a UF ratio of 0.5 in all the loadings, when compared to the particles produced at the UF ratio of 1.0. The matrix produced at a U/F ratio of 1.0 is less rigid and, therefore, the release rates for the active agents are higher (Figure 7). At 15 wt% of drug loading the encapsulation efficiency of PMNs lies between 52- 67% presented in Table 1.

**Effect of 5-Fluorouracil Content**
Figure 8 shows the release of 5-FU, suggesting that the PMNs containing 25 w/v% 5-FU exhibited faster release behaviour than those containing 5 w/v% 5-FU in the formulations. Being independent of preparation method, the PMNs showed controlled release of 5-FU. At a loading of 25 w/v% 5-FU, 85% of the loaded amount was released after 15 h. With a loading of 5 w/v%, about 60% of the 5-FU was released at the same time. It is noticed that faster release rates have

<table>
<thead>
<tr>
<th>Sample code</th>
<th>$k$</th>
<th>$n$</th>
<th>Correlation coefficient ($r$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS-1</td>
<td>0.021</td>
<td>0.480</td>
<td>0.983</td>
</tr>
<tr>
<td>MS-2</td>
<td>0.032</td>
<td>0.475</td>
<td>0.973</td>
</tr>
<tr>
<td>MS-3</td>
<td>0.045</td>
<td>0.460</td>
<td>0.980</td>
</tr>
<tr>
<td>MS-4</td>
<td>0.047</td>
<td>0.528</td>
<td>0.954</td>
</tr>
<tr>
<td>MS-5</td>
<td>0.018</td>
<td>0.435</td>
<td>0.977</td>
</tr>
<tr>
<td>MS-6</td>
<td>0.037</td>
<td>0.443</td>
<td>0.995</td>
</tr>
<tr>
<td>MS-7</td>
<td>0.024</td>
<td>0.484</td>
<td>0.983</td>
</tr>
<tr>
<td>MS-8</td>
<td>0.015</td>
<td>0.528</td>
<td>0.962</td>
</tr>
</tbody>
</table>
been observed for formulations that contain higher amount of 5-FU at 0.50 U/F ratio in the matrix.

**Effect of Graft Copolymer Content on PMNs**

In the present research, UF was employed to cross-link CS-PMNs. However, chitosan and chitosan-\(g\)-acrylamidoglycolic acid (CS-\(g\)-AGA) copolymer matrices gave uniform size PMNs probably because UF could only cross-link with CS, but not with AGA. However the graft copolymers are showing the slow release rates as compared to pure CS. In the case of encapsulation efficiency, 5-FU formulations with loadings ranging 61-76% could be achieved at different copolymer compositions. The encapsulation efficiency data are given in Table 1.

To investigate the effect of pH and ionic strength of the external medium on the swelling of PMNs, we have measured the percentage cumulative release in both pH 1.2 and 7.4 media. Cumulative release data presented in Figures 7, 8, and 9 indicate that at the first two hours the cumulative release was very low in pH 1.2, but, at higher pH 7.4 a considerable increase in the cumulative release is observed for all PMNs. At higher pH (above the pK\(_a\) of the microgels), the -COOH groups may dissociate, increasing the osmotic pressure inside the microgels, resulting in higher swelling. Cumulative release in the both pH media depends upon the extent of cross-linking.

The effect of AGA composition in the matrix of CS and AGA was studied at a constant drug loading of 15 wt%, wherein it was found that CS-\(g\)-AGA (e.g., MS-8 sample) PMNs produced almost 100% cumulative release in about 11 h, whereas they produced almost increasing amount of AGA, probably due to the loose cross-linked chains of AGA in the PMNs. Microscopically speaking, there is a relaxation response of the polymer chains because of the stresses introduced during the process of dissolution, resulting in an increase of dimension (radius of gyration) of the polymer coil. Thus, a significant increase in molecular volume of the overall hydrated polymer matrix took place due to increased swelling of AGA component of the copolymer. It is obvious that by increasing the free volume of the system, the diffusion of drug would be increased as well.

**CONCLUSION**

The synthesis of PMNs and their drug release properties have been carried out successfully by the CS and its graft copolymers cross-linked with UF in acidic media at room temperature to study the
controlled release of 5-FU. Indeed, the matrices prepared could offer a wide array of release patterns and rates. The remarkable advantage of this hydrophilic polymeric system is that it is solely made of chitosan and its copolymers with AGA, which are non-toxic, and biodegradable. All these CS-PMNs copolymers were obtained under mild conditions without any surfactants and were stable under acidic and neutral media. The preliminary results of 5-FU loading and release experiments indicate that this system seems to be a very promising vehicle for the administration of hydrophilic drugs. The release of drug was controlled by penetration of external medium into the matrix or by drug diffusion into the matrix pores or by both. SEM, DSC and XRD studies of 5-FU loaded CS-PMNs have shown molecular dispersive level of the drug in the matrices.

ACKNOWLEDGEMENT

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